

As a manuscript



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**ETHANOL AND GENERAL ANESTHESIA EFFECTS ON CORTICAL
ACTIVITY IN THE PRIMARY SOMATOSENSORY CORTEX OF THE
NEWBORN RAT BRAIN**

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ABSTRACT

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GENERAL DESCRIPTION OF THE WORK

The research topic relevance

Currently, the study of the characteristics of the developing brain and factors affecting the normal functioning of neuronal networks in ontogenesis is an urgent problem of neurophysiology. It is known that the physiological apoptosis lasting during the critical period of the nervous system development is a necessary condition for the formation of neural connections, pathways and structures (Buss et al., 2006; Oppenheim et al., 1991). At the same time, massive neurodegeneration caused by various damaging factors can lead to both morphological disturbances and long-term functional changes in the brain and behavior as a result (Rohn et al., 2009; Ribe et al., 2008; Mattson et al., 2006; Viswanath et al., 2001). A growing body of evidence accumulated over the past decades reveals that several classes of drugs that pregnant women sometimes abuse, such as alcohol, ketamine and benzodiazepines, as well as certain classes of medicines that are commonly used in pediatrics and pregnant women, including general anesthetics and antiepileptic medicines at early stages of development can have dangerous secondary effects, including massive programmed death of brain cells (Creeley et al., 2013; Léveillé et al., 2010; Patel et al., 2009; Jevtovic-Todorovic et al., 2003; Ikonomidou et al., 2000). Thus, the impact of ethanol on the stage of prenatal human development can lead to a variety of neurobehavioral defects clinically united into fetal alcohol spectrum disorders that can be diagnosed in early childhood with its symptoms often persist throughout life (Sadrian et al., 2013). Fetal alcohol syndrome is the most severe condition, comprising typical craniofacial defects, growth retardation and abnormal development of the nervous system (Jones et al., 1973).

The first week of life in rodents is an early stage of "rapid brain development", during which the morphological and functional differentiation of neurons occur at the fastest rate, as well as the formation of synaptic connections (Ben-Ari et al., 2001). This period approximately corresponds to the period from 20 to 30 weeks of the human fetus gestation (Clancy et al., 2007; Clancy et al., 2001; Dobbing et al., 1979). Physiological activity in the developing brain is characterized by unique patterns of electrical activity (Colonnese et al., 2012; Blankenship et al., 2010; Khazipov et al., 2006). In rats during the first postnatal week cortical brain activity is organized in oscillatory activity bursts in alpha-beta (spindle-bursts) and gamma frequency ranges (Yang et al., 2013; Minlebaev et al., 2011; Yang et al., 2009; Khazipov et al., 2004) that are internally generated in neuronal thalamocortical circuits, but they can also be triggered by inputs from the sensory periphery (Khazipov et al., 2004). Similar patterns of activity are also specific for the human brain during the second half of fetal development

(Colonnese et al., 2010; André et al., 2010; Milh et al., 2007). The brain of newborn rodents in this time period as well as in utero developing brain of primates is particularly sensitive to apoptotic effects of ethanol and general anesthesia (Olney, 2014; Creeley et al., 2013).

It is known that early neuronal activity not only plays a role in synaptic plasticity, but also promotes the survival of neurons, preventing apoptosis in the development process (Léveillé et al., 2010; Heck et al., 2008; Mennerick et al., 2000). Indeed, substances that inhibit N-methyl-D-aspartic acid (NMDA) receptors and enhance the functions of gamma-aminobutyric acid (GABA) receptors caused massive apoptosis in the brain of newborn rodents and in primates during prenatal development (Olney, 2014; Jevtovic-Todorovic et al., 2003; Ikonomidou et al., 2000; Ikonomidou 1999).

Despite the intensive studies of ethanol and general anesthetics action targets, there are no data revealing the effects of these drugs on the electrical activity of the brain during the early period of development *in vivo*. Taking into account the critical impact of neuronal activity on survival and normal formation of synaptic connections, the analysis of the neurotoxic effect of ethanol and general anesthesia on the electrical activity of the developing brain is undoubtedly an urgent task of neurobiology of development.

The purpose of the study – to reveal the influence of ethanol and general anesthesia on spontaneous and sensory evoked electrical activity in the rat primary somatosensory cortex.

In accordance with the purpose following tasks were set:

- 1) To study the age and concentration dependence of ethanol effects on electrical activity and apoptosis in the rat primary somatosensory cortex.
- 2) To characterize the effects of inhalational anesthetic isoflurane on spontaneous and sensory evoked electrical activity of the rat primary somatosensory cortex during development.
- 3) To reveal the effect of combined administration of general anesthetics ketamine and midazolam on the electrical activity of the rat primary somatosensory cortex during the first week of life.

Scientific novelty of the work

In the present study it was shown that ethanol inhibits spontaneous activity and sensory evoked oscillatory bursts of activity in the primary somatosensory cortex of newborn rats during the first week after birth. In the same period of development ethanol-induced apoptosis reaches its peak. Thereby, the correlation of these electrophysiological experiments with the concentration dependence of the apoptogenic effects of ethanol is observed. It was also discovered that general anesthetics – isoflurane, ketamine and midazolam inhibit electrical activity in the

primary somatosensory cortex of newborn rats. It was shown that the effects of isoflurane are qualitatively different in animals of the first week of life and in more adult rats. Thus, during the first postnatal week, isoflurane completely inhibited the spontaneous electrical activity of the cortex and suppressed the sensory evoked early oscillatory patterns, while in older animals, the anesthetic caused specific burst-suppression patterns alternating with activity inhibition periods. Moreover, it was shown that combined administration of ketamine and midazolam in doses equivalent to those used for surgical anesthesia causes substantially complete suppression of the spontaneous electrical activity, as well as a significant decrease in the oscillatory component of the sensory stimulation response in the somatosensory cortex of neonatal rats.

Scientific and practical significance of the work

The obtained data are of both fundamental and practical importance. Since the period of the first week after birth in rat approximately corresponds to the period from 20 to 30 weeks of the human fetus gestation (Clancy et al., 2007; Clancy et al., 2001; Dobbing et al., 1979), the brain of the newborn rodents in this time interval, as well as the prenatal developing brain of primates, is particularly sensitive to the apoptogenic effects of ethanol and general anesthesia. The results reveal the causes of neurotoxic effects of prolonged exposure to ethanol and anesthetics in the early stages of development, accompanied by the emergence of long-term neuropsychiatric disorders. The present study provides evidence to support the hypothesis that the basis for the developmental disorders of the nervous system resulting from the effects of ethanol and general anesthetics may be the suppression of electrical activity, which is a mechanism that stimulates apoptosis and leads to significant amounts of neuronal death.

Besides, the received data may be of importance for practical medicine. As anesthesia procedures become more complex and time-consuming, minimizing the risks arising from the use of general anesthetics is one of the most important aspects in clinical practice. The obtained results suggest the possibility of carrying out further translational studies of the general anesthetics influence on brain activity of the fetus and premature newborns that will keep track of potentially dangerous effects on fetus and newborn health. Similarly, the inhibitory ethanol effect on the electrical activity of the neonatal brain, correlated with its apoptotic impact, revealed in the present study, suggests the possibility of evaluating the potentially damaging action of alcohol on the brain of the fetus and the newborn by analyzing changes in the parameters of cortical brain activity.

Research methods

The electrical activity of the rat brain that appeared spontaneously and in response to sensory stimulation was studied using electrophysiological intracortical

recordings *in vivo*. Extracellular recording of activity in the area of forelimbs and whiskers representations of the rat primary somatosensory cortex was carried out using multichannel silicon electrodes. To characterize the neuroapoptotic actions of ethanol during rat postnatal development and apoptotic destruction of neuron nuclei cleaved Caspase-3 immunohistochemistry and DAPI containing Fluoromount were used.

The reliability of received data

The reliability of the obtained data is based on a large amount of experimental research results with adequate methodological approaches and statistical processing of the results.

The main scientific theses for the defense:

1) Ethanol has inhibitory effects on the electrical activity of the primary somatosensory cortex of rat brain, which are characterized by concentration and age dependencies. The suppression of cortical activity is most pronounced in the period of the first postnatal week, which correlates with the degree of apoptotic neurodegeneration resulting from the action of ethanol.

2) Isoflurane completely inhibits the electrical activity of the primary somatosensory cortex of rats during the first postnatal week, while the effects of the anesthetic change during development from complete reduction to the initiation of burst-suppression patterns alternating with activity inhibition periods.

3) General anesthesia with combined administration of ketamine and midazolam leads to nearly complete suppression of electrical activity in the primary somatosensory cortex of newborn rats.

The author's personal contribution to the study

The data given in the work were obtained with the personal participation of the applicant at all stages of the work, including the development of a research plan, the performance of experiments, data analysis and publications design.

BASIC CONTENT OF THE WORK

Materials and methods of research

Preparation of the animal. The experiments were carried out in accordance with standards adopted by the Institutional Animal Care and Use Committee of Kazan State Medical University (N9-2013). Wistar rats of either sex from postnatal day P1 (P0 = day of birth) till postnatal day P69 were used. The scheme of the experiment is shown in Figure 1. During the surgical procedure inhalation anesthetic isoflurane was used. During recording the fixation of the animal's head was carried out by attaching the bars to the frame of the stereotaxic apparatus. The chlorinated silver wire was used as a ground electrode, which was placed in the visual cortex or cerebellum.

The data presented for each isoflurane (Baxter Healthcare, USA) (0.5-2%) concentration were acquired no less than 15 min after a change in isoflurane concentration. During the study of the effects of 20% ethanol ("Hippocrates", Russia), ketamine (Merial, France) and midazolam (Sigma-Aldrich, USA) on the activity of the primary somatosensory cortex (S1) of newborn rats, injections of test substances were made intraperitoneally (IP) in concentrations corresponding to the experimental protocols. Physiological saline was used in control experiments and its injection had no significant effect on the cortical activity of rat brain.

Extracellular recording. The registration of local field potentials and multiple unit activity was performed using multichannel linear silicon electrodes (Neuronexus Technologies, USA) with 16 or 32 recording sites (15 μm diameter, 100 μm separation distance between recording sites) (Fig. 1 B). The electrode was placed vertical into the S1 – to the representation of whiskers or forelimbs to a depth of 1.5 mm to trace the columnar activity from all cortical layers. Spontaneous movements were recorded by piezoelectric detectors (Fig. 1 A).

The sensory stimulation was performed using the 8-channel electric stimulator Master-8 (AMP Instruments, Israel) and the ISO-Flex adapter (AMP Instruments, Israel) by deflecting the whisker in the direction opposite to its growth, or short-term (10 ms) touching the surface of the skin with a rod attached to the piezoelectric actuator. The topography zone of the skin or the principal whisker corresponding the cortical column representation was determined by the minimum latent period (delay) of the multiple unit activity that appeared in the fourth layer (L4) in response to stimulation.

In P2-7 rats under control conditions a short (10 ms) deflection of the principal whisker or a touch to the topographic area of the skin of the forelimb caused a specific oscillatory response in the corresponding S1 zone, consisting of a sensory evoked potential followed by gamma (30 -80 Hz) and spindle-burst (8-30

Hz) oscillations (Fig. 1 C). Sensory evoked potential and subsequent sensory evoked oscillations were accompanied by maximum deviations of the local field potential in L4, where the depolarization regions of most neurons and the highest density of multiple unit activity during the induced oscillatory patterns were also observed (Fig. 1C). In addition, it should be noted that there was a significant correlation between neuronal activity bursts and animal movements.

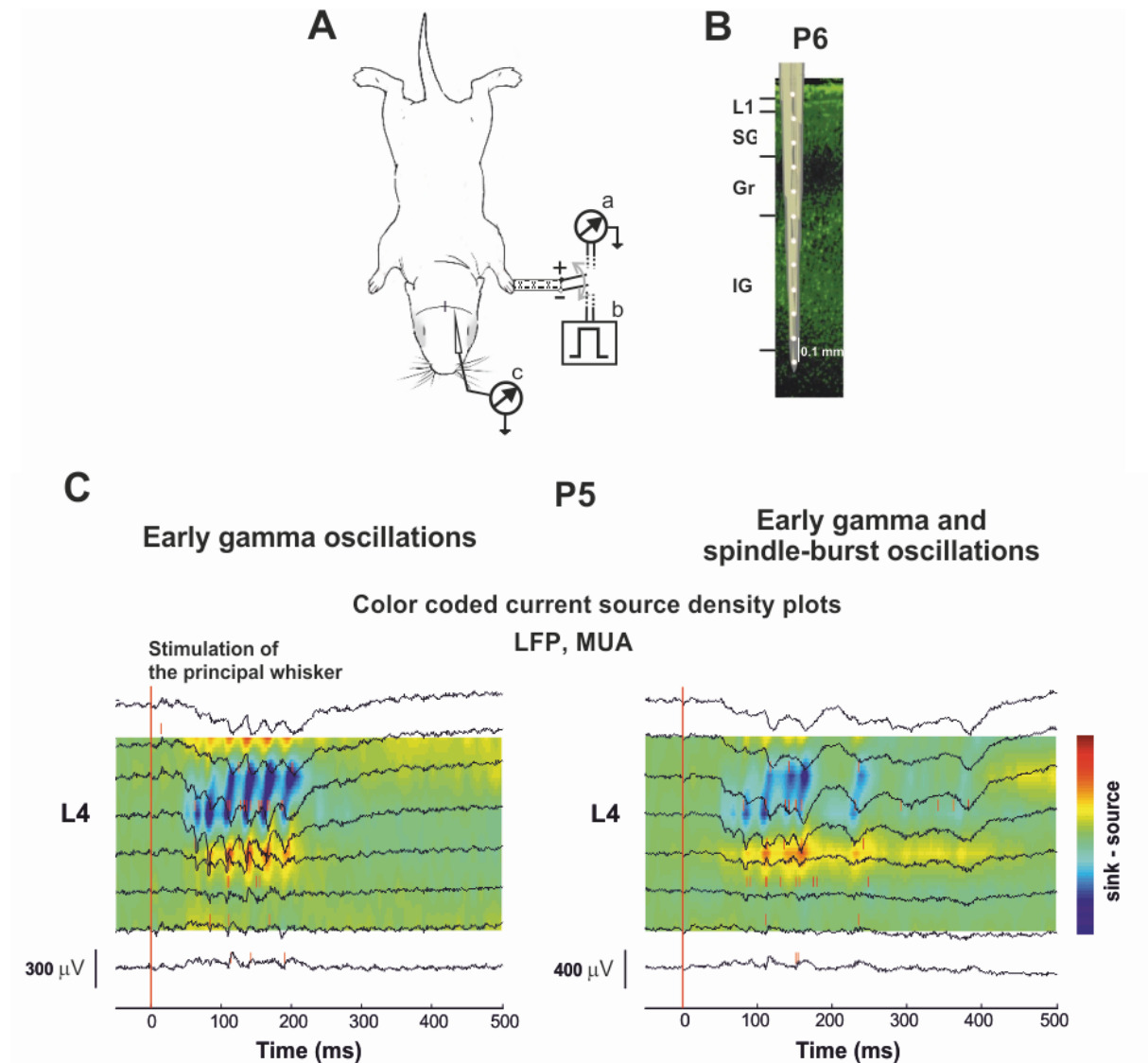


Figure 1 – The scheme of the experiment and examples of early cortical oscillatory patterns of the brain activity. (A) The experiment scheme for electrical activity recording in the S1 of the newborn rat. The scheme shows: (a) the piezoelectric detector that records the movements of the animal; (b) the stimulator; (c) the amplifier. (B) The multichannel electrode overlaid on brain slice of a P6 rat. S1 layers are marked on the slice: the first (L1), supragranular (SG), granular (Gr) and infragranular (IG). (C) Examples of oscillatory patterns of early cortical brain activity in the P5 newborn rat. Caused by stimulation of the principal whisker sensory evoked responses included sensory evoked potentials, as well as early gamma (30-80 Hz) oscillations (left) and a combination of early gamma and spindle-burst (8-30 Hz) oscillations (right). LFP – local

field potentials – black traces; MUA – multiple unit activity – red vertical lines overlaid on a color coded plot of the current source density at different depths of the cortical column. Sink – the area of the outflow of extracellular positive charges (membrane depolarization) – displayed by gradations of blue color; source – the area of the inflow of extracellular positive charges – displayed by gradations of red color.

The electrical signals were amplified and filtered (10000X, 0.5 Hz - 9 kHz bandwidth) using a 128-channel Digital Lynx SX amplifier (Neuralynx, USA) or a non-serial amplifier (A. Alekseev, Russia). The signals were then digitized at a sampling rate of 10, 25 or 32 kHz (the resolution voltage of 0.5 mV) and stored for later analysis. Spontaneous and sensory evoked activity was recorded for 1 hour, after that isoflurane, or a combination of ketamine and midazolam (IP) or ethanol (20% in physiological saline, IP) were administered. Further a recording of spontaneous and sensory evoked activity was carried out for 3-4 hours.

Data analysis. The data was preliminarily processed using custom-developed suite of programs in Matlab analysis environment (MathWorks, USA). To detect multiple unit activity the original signal was filtered in the range of 300-3000 Hz. Action potentials (units) were negative polarity deviations of the local field potential, exceeding in the amplitude of 3-5 dispersions from the baseline, depending on the age of the animal. In all figures the positive polarity of the signals is shown as an upward deflection. Sensory evoked potential was defined as the first stimulus following deviation of the potential in the L4.

To analyze sensory evoked oscillations (bursts of electrical activity), the 500 ms recording period following the sensory evoked potential was used. As base level of activity, the recording section preceding the stimulus was evaluated, which duration was 200 ms. Local field potentials and multiple unit activity were detected and analyzed using author programs in the Matlab environment. The spectral analysis was carried out using the Chronux toolbox procedures for Matlab.

Gamma and spindle-burst oscillations were detected using the following steps: 1) LFP signal was bandpass filtered (30–80 Hz for gamma and 8–30 Hz for spindle bursts); 2) times of detected activity having amplitude of the oscillations exceeded the background by 3-5 times; 3) gamma and spindle-burst oscillations were considered as minimum three cycles with periods less than 30 ms and 120 ms each, respectively, both associated with action potentials.

Statistics. Statistical analysis was performed using the tools of the Matlab package. The correspondence of the obtained data set to the normal distribution was determined using the Kolmogorov-Smirnov test.

The comparison of samples for normally distributed data was performed using ANOVA method, in case of abnormally distributed data the two-side

Wilcoxon rank sum test was used. The data are presented as the mean \pm standard error, either as a median and 25 and 75 percentile (median [25% -75%]). A statistical probit analysis was used to detect a semilethal dose of ethanol (LD50).

Determination of toxic doses and concentrations of ethanol in the blood.

To determine the toxic doses of ethanol 78 rats aged P1–7 were used. Ethanol (20% in normal saline) was administered intraperitoneally to 11 groups of animals at doses of: 2.5; 3.7; 6; 6.7; 7.4; 8.2; 9; 11; 12.5; 13.7; and 15 g/kg. Animals were monitored for 6 h after ethanol administration. Probit analysis revealed that LD50 of ethanol is of 11.45 g/kg (confidence interval 10.59–12.26 g/kg) and the 95%-lethal dose is 14.26 g/kg (confidence interval 13.22–16.46 g/kg). None of the animals died after ethanol administration at doses below 9 g/kg.

Blood alcohol concentrations (BAC) were determined in 25 rat pups of postnatal age P5–7. Alcohol was administered intraperitoneally (20% in normal saline) at doses of 1 g/kg, 1+3 g/kg with a 1 h interval, and 6 g/kg (n = 8 animals per dose). Control animals were administered with an equal volume of normal saline (IP). Blood ethanol concentrations were determined 1 and 4 hours after injection at the Bureau of Criminal and Medical Expertise of the Ministry of Health of Tatarstan Republic using a Cristallux-4000M gas chromatograph (Meta-chrom, Russia). The chemical study of the blood of newborn rats showed that when the dose was 1 g/kg, the concentration of ethanol in the blood averaged: 1 hour after the administration – 36 mg/dl, after 4 hours – 4.6 mg/dl; when the dose was 1+3 g/kg the concentration of ethanol in the blood averaged: 1 hour after the administration – 266 mg/dl, after 4 hours – 237 mg/dl; when the dose was 6 g/kg the concentration of ethanol in the blood averaged: 1 hour after the administration – 355 mg/dl, after 4 hours – 388 mg/dl ($p < 0.05$, $n = 16$). It was shown that ethanol-induced apoptosis occurs at ethanol blood levels above 200 mg/dl maintained for 4 hours and that the apoptogenic effect of ethanol is enhanced and distributed proportionally to the time during which its concentration in the blood is remained above this level (Ikonomidou et al., 2000).

Identification of ethanol neuroapoptogenic effects in the rat primary somatosensory cortex. Brain slices were incubated in phosphate buffered saline (PBS) containing the primary antibodies against Cleaved Caspase-3 (ASP 175) (Cell Signalling Technology Inc., USA) diluted 1:1000, and then incubated in PBS containing the secondary fluorescent antibodies Cy3 AffiniPure Donkey Anti-Rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories Inc., USA), dilution 1: 1000. After incubation the slices were rinsed in water and mounted with a DAPI (4.6-diamidino-2-phenylindole dihydrochloride) containing Fluoromount (Vector Laboratories Inc., USA).

RESULTS

1. Ethanol effects on electrical activity and apoptosis in the rat primary somatosensory cortex

1.1 Ethanol effects on the spontaneous electrical activity of the rat primary somatosensory cortex

During the first postnatal week, the spontaneous activity in the S1 was characterized by intermittent activity bursts occurring with an average [25–75% quartile range] frequency of 2.7 [0.6–5.2] min⁻¹ and included oscillations in gamma and alpha-beta frequencies associated with multiple unit activity (P1–7, n = 14) (Fig. 2 A). Injection of ethanol (6 g/kg, IP) in rat pups resulted in a rapid and almost complete suppression of cortical activity. In P5–7 rats, in the presence of ethanol both gamma and spindle-bursts were completely eliminated (Fig. 2). Ethanol-induced suppression of the early activity bursts was associated with a dramatic reduction in the spontaneous multiple unit activity frequency from 1.40 [0.80–2.40] to 0.04 [0.02–0.14] s⁻¹ 1 h after ethanol administration. Ethanol also strongly suppressed limbs/body movements with a decrease in the movement frequency from 8.4 [5.8–11.5] to 0.7 [0.3–1.3] min⁻¹ (Fig. 2 D). Profound suppression of cortical activity bursts, multiple unit activity and limbs/body movements in neonatal rat pups occurred within few minutes after ethanol administration and persisted through the entire 4-h-long electroencephalogram (EEG) recording period.

To analyze the dose-dependence in the inhibitory effects of ethanol on S1 activity in P4–7 rats the following concentrations were used: 1 g/kg, 1+3 g/kg with a 1-h interval, 4.5 and 6 g/kg. At a dosage of 1 g/kg (P4–5, n = 6), ethanol exerted nearly 2-fold suppressive effects on S1 multiple unit activity with a similar inhibition of the occurrence of spindle bursts and gamma oscillations (to 58 [49–79] % and 56 [34–90]%, respectively, without any change in the oscillation power), and on the frequency of movements (Fig. 2). Ethanol at higher doses, 1 + 3 g/kg with a 1 h interval and 4.5 g/kg produced strong suppression of S1 activity including a decrease in multiple unit activity to 18 [4–27]%, burst frequency to 8 [5–29]% with a similar suppression of spindle-burst occurrence to 8 [2–23]% and gamma oscillations to 7 [5–20]%, a decrease in corresponding alpha-beta and gamma oscillation power to 9 [7–22]% and 33 [25–54]%, respectively, and movement frequency to 16 [12–29]% (the pooled data for 1+3 and 4.5 g/kg) (P4–6, n = 11). The most robust effect was suppression of activity by ethanol at 6 g/kg. The results of experiments in which this concentration was used, showed suppression of multiple unit activity attained 2.8 [2.3–6.1]%, burst frequency

attained 0.01 [0.0–3.6]% and movement frequency attained 8.5 [4.0–15.3]% of control levels (n = 9) (Fig. 2).

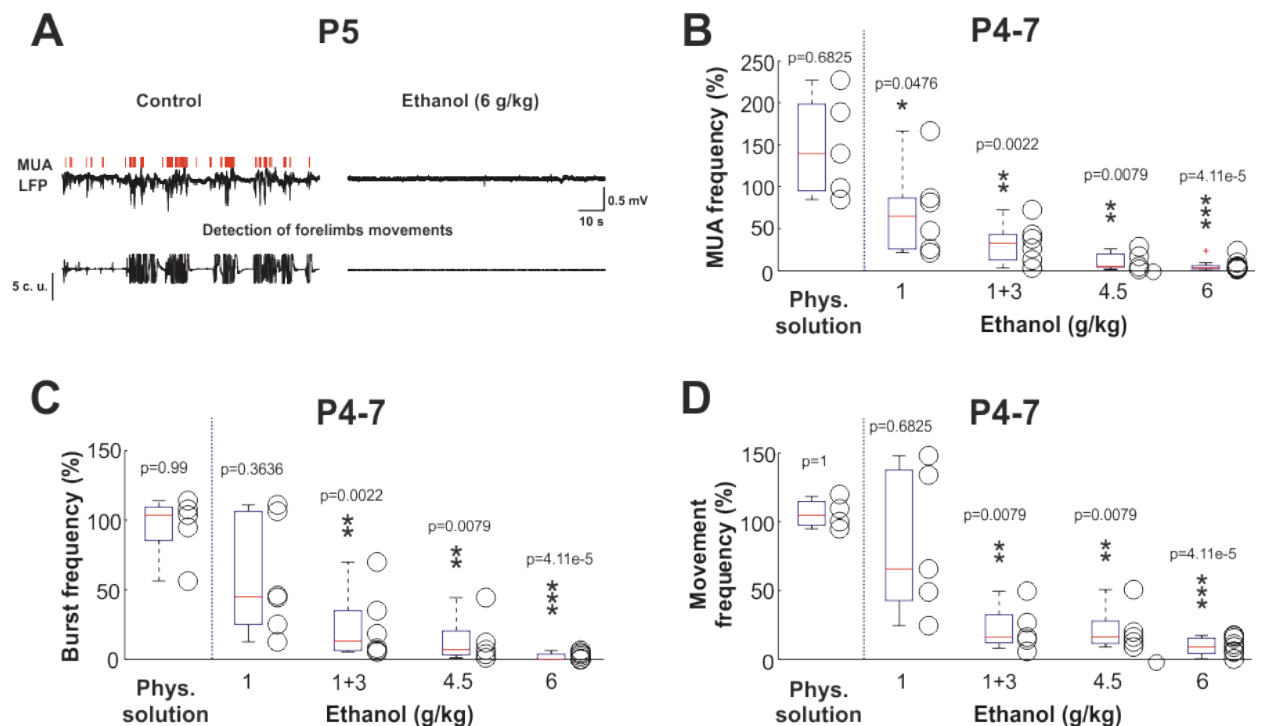


Figure 2 – Effects of ethanol on the spontaneous activity in the S1 of P4-7 neonatal rats. (A) Example traces of the spontaneous electrical activity in the L4 of the forelimbs representation area in the S1 and mechanogram of the motor activity of the P5 rat (LFP – local field potentials – black traces; MUA – multiple unit activity – red vertical lines above; c. u. – conventional units) in control conditions (left) and 10 min after ethanol injection at a concentration of 6 g/kg (20%, IP) (right). (B) The influence of physiological solution and ethanol on the frequency of multiple unit activity. (C) The effect of saline and ethanol to the frequency of bursts. (D) The impact of physiological solution and ethanol on the motor activity. Each open circle corresponds to an individual animal and boxes indicate median and error during 15 min recording period normalized to control values prior to physiological solution (IP) or ethanol (20%, IP) injection. (B-D) The pooled data obtained in the area of forelimbs and whiskers representations of P4-7 rats (n = 27) 1 hour after the injection are presented.

To determine the developmental changes of the ethanol influence, the effects of ethanol on cortical activity in the S1 of P13-P23 rats were studied. In control conditions spontaneous cortical activity switched from discontinuous to continuous temporal organization after the first postnatal week along with a disappearance of the early activity patterns including spindle-bursts and early gamma oscillations. The amount of spontaneous limbs/body movements decreased with age in keeping with a developmental disappearance of myoclonic twitching. In this age group ethanol (6 g/kg) slowed down the ongoing EEG activity evoking slow delta-wave

oscillations and reducing neuronal firing from 14.8 [11.7–17.0] to 2.4 [2.2–4.9] s⁻¹ (P13–23, n = 5; p = 0,008). Ethanol inhibited the frequency of the spontaneous limbs/body movements from 1.50 [1.39–1.90] to 0.37 [0.07–0.49] min⁻¹ (p = 0.008).

1.2 Effects of ethanol on the sensory evoked electrical activity of the rat primary somatosensory cortex

In control conditions a brief mechanical stimulation of the topographic spot at limbs or a deflection of the principal whisker evoked complex S1 responses consisting of the initial sensory evoked potentials followed by gamma and spindle-burst oscillations associated with multiple unit activity. The effect of ethanol on the sensory evoked responses in the S1 has been investigated in several age groups of animals. In P1-2 rats ethanol almost completely suppressed the sensory evoked bursts. In P4-7 rats ethanol (6 g/kg) affected neither amplitude (control: 536 [437–879] μ V; ethanol: 434 [350–724] μ V; p = 0.22; n = 10) nor onset latency of the sensory evoked potentials (control: 36 [34–41] ms; ethanol: 41 [34–45] ms; p = 0.22; n = 10) (Fig. 3). Although multiple unit activity during the sensory evoked potentials showed a tendency to decrease after ethanol administration, this was not significant. Ethanol suppressed the sensory evoked oscillatory component of cortical bursts (Fig. 3 A).

During development sensory evoked responses switched from bursting to acuity with a disappearance of sensory evoked oscillatory bursts. The sensory evoked responses were moderately affected by ethanol in P13–23 rats including a decline in amplitude, increase in latency of the sensory evoked potentials and a reduction in multiple unit activity during and after the sensory evoked potentials.

Thus, during the first week of life ethanol has an inhibitory effect on the spontaneous activity, and also suppresses the sensory evoked oscillations in dose-dependent manner. When exposed to rats aged P13-23, ethanol at a concentration of 6 g/kg inhibits the continuous background activity of the cortex, causing slow delta wave oscillations and decreasing the activity of neurons in all layers.

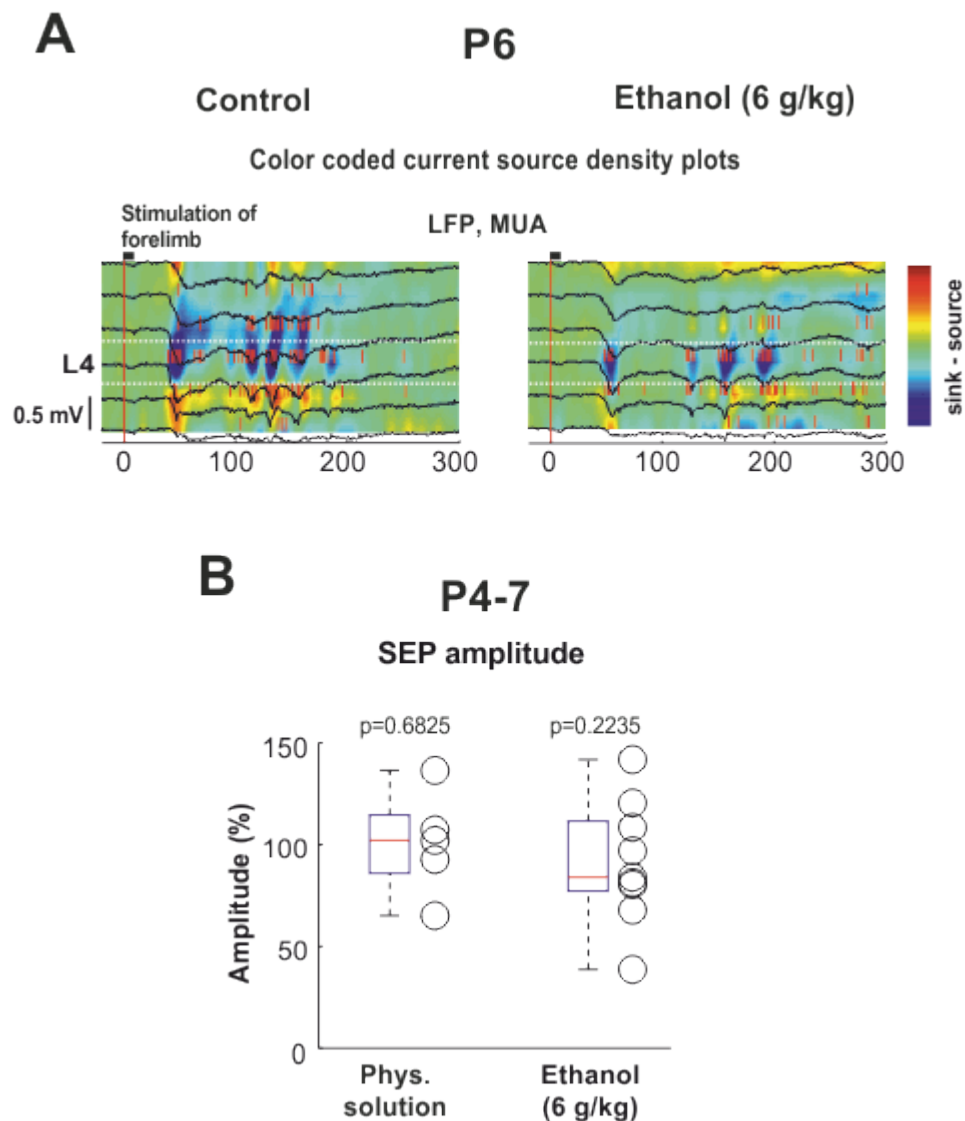


Figure 3 – Ethanol (6 g/kg) effects on the sensory evoked activity in the S1 of neonatal rats. (A) Example traces of the sensory evoked responses caused by mechanical stimulation of the forelimb at different cortical depths in the area of forelimbs representation in the S1 of the P6 rat (LFP – local field potentials – black traces; MUA – multiple unit activity – red vertical lines overlaid on the color coded current source density plot) under control conditions (left) and 1 hour after injection of ethanol at a concentration of 6 g/kg (20%, IP) (right). Stimulus onset is indicated by vertical red line. L4 borders are marked by white dashed lines. (B) The impact of physiological saline and ethanol (6 g/kg) on the parameters of the sensory evoked responses in the S1 of newborn rats. Each circle corresponds to an individual rat. The pooled data of the physiological saline (IP) and ethanol (20%, IP) influence, normalized to control values, obtained in the area of forelimbs and whiskers representations in the S1 of P4-7 rats 1 hour after the administration are presented ($n = 10$).

1.3 The investigation of the ethanol influence on a neuronal apoptosis in the rat primary somatosensory cortex

In these experiments, neuroapoptogenic effects of ethanol depending on concentrations and animals ages were studied. Apoptosis of neurons was assessed by cleaved caspase-3 staining of brain sections (Olney et al., 2002). The present study showed that few neurons of the S1 undergo apoptosis during the postnatal period under control conditions (17 ± 7 cleaved caspase-3 stained neurons/mm³) (P1–7; n = 7).

To study age-dependence of the ethanol-induced apoptotic neurodegeneration rats were administered with ethanol (20%, IP) at 6 g/kg (P1–P17; n = 32). 8 h after ethanol administration (Olney et al., 2002) the staining of S1 cortical sections was performed to determine the presence of cleaved caspase-3 indicating ethanol-induced apoptosis in the cerebral cortex. In addition, the sections were stained with a chromatin-binding DAPI dye. The images were obtained with a confocal microscope. Cleaved caspase-3 stained neurons were characterized by various degrees of morphological degeneration and nuclear damage. Many of cleaved caspase-3 stained neurons displayed chromatin condensation with a formation of the DAPI-stained apoptotic bodies that were also evident as shadows in caspase-3 images. In order to quantify neuroapoptosis cleaved caspase-3 stained cells retaining clear neuronal morphological features with dendritic arborizations were only counted.

Ethanol-induced apoptosis in the S1 was characterized by a bell-shaped developmental profile (Fig. 4 B) with mild effects observed at P1 (119 ± 63 neurons/mm³; n = 3), and a progressive increase in apoptosis with age, with a peak attained at P7 (1235 ± 284 neurons/mm³; n = 3), and further developmental decline to 82 ± 35 neurons/mm³ at P14–17 (n = 3). Cleaved caspase-3 stained neurons were observed in all cortical layers.

Dose-dependence of the ethanol-induced neuroapoptotic effects in the S1 was studied in P5 rats at 4 dosage regimens: 1 g/kg, 1 and 3 g/kg with a 1-h interval, 4.5 and 6 g/kg (Fig. 4 C). Rats introduced with normal saline (IP) in a volume equivalent to injectable ethanol served as controls. At a dose of 1 g/kg (20%, IP), which resulted in an increase in BAC levels to 47.3 ± 10.8 mg/dL 1 h after ethanol administration and returned to nearly zero after 4 h, a slight tendency to increase in the number of cleaved caspase-3 stained neurons was found and it was not significant. Administration of ethanol by 2 consecutive injections of 1 and 3 g/kg with 1 h interval caused an increase in BAC levels to 350 ± 89 mg/dL (n = 4) and 311 ± 52 mg/dL (n = 4) 1 and 4 h after the first ethanol injection, respectively, and prominent apoptosis attaining 74 ± 11 neurons/mm³ (n = 3).

Similar apoptosis levels were attained at 4.5 g/kg (75 ± 9 neurons/mm³) (n = 3) (Fig. 4 C). Massive apoptosis was observed at 6 g/kg ethanol (728 ± 92 neurons/mm³) (n = 3) (Fig. 4 C), which caused sustained BAC levels above 300 mg/dL. These findings are in general agreement with the observation that ethanol-induced apoptosis occurs at ethanol blood levels above 200 mg/dL maintained for several hours (Ikonomidou et al., 2000).

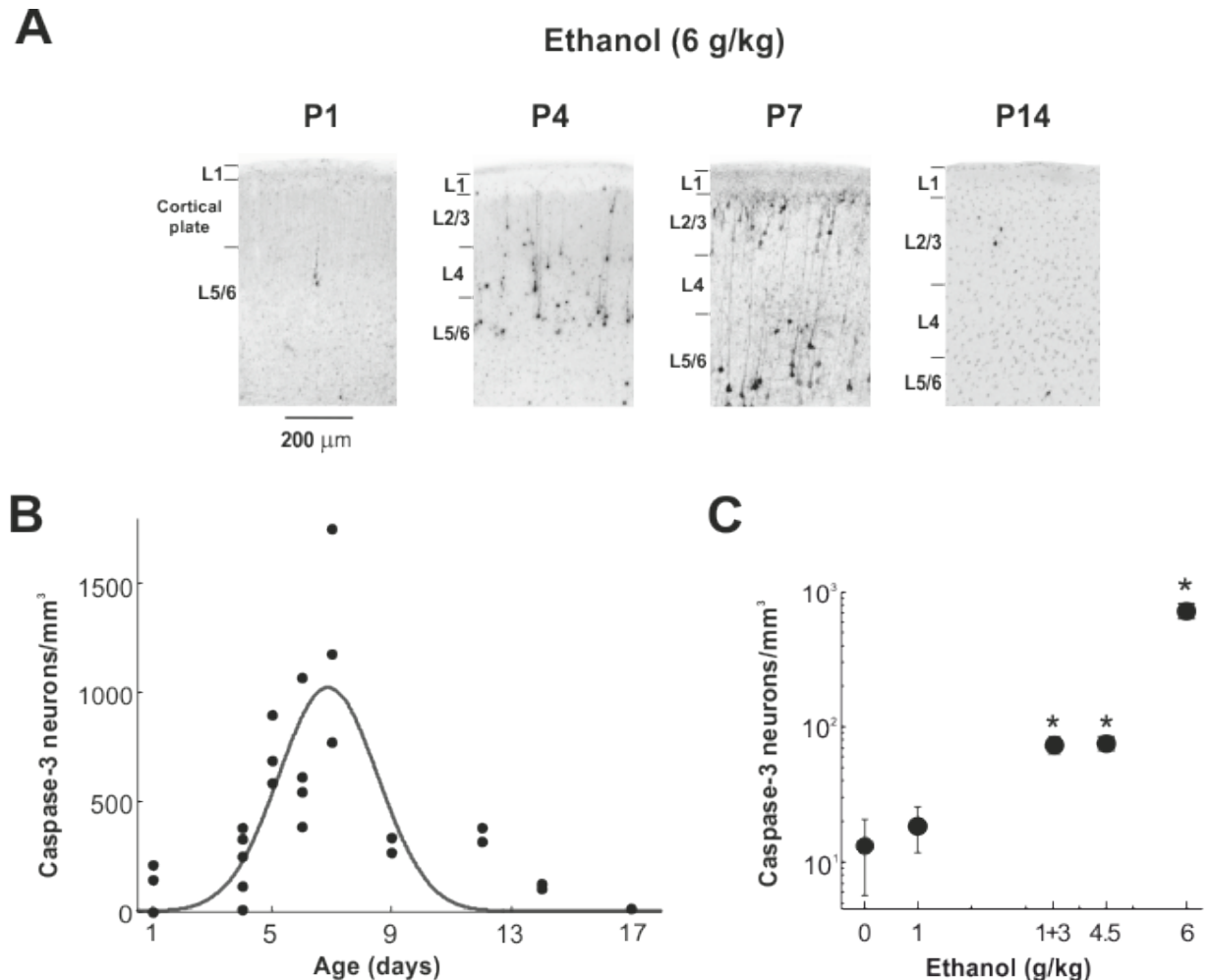


Figure 4 – Age and concentration dependences of ethanol-induced apoptotic processes in the whiskers representation area in the S1 of rats. (A) Histological sections of the S1 of rats at different postnatal ages that were made eight hours following treatment with ethanol (20%, IP). The sections have been stained with antibodies to cleaved caspase-3. (B) Age dependency of the total density of cleaved caspase-3 stained neurons treated with ethanol. Each point corresponds to an individual animal (n = 32). A peak of ethanol-induced apoptotic neurodegeneration in the rat S1 was revealed at around P7. (C) The dependence of the total density of caspase-3 stained neurons on the ethanol dose. The pooled data obtained from the whiskers representation area in the S1 of P5 rats are presented (n = 15).

Thus, ethanol (20%, IP) at a concentration of 6 g/kg causes a massive apoptosis of the rat S1 neurons, which is most pronounced during the first week of life that corresponds to the inhibitory effect of this substance on the cortical activity during this period.

2. Effects of general anesthetics on the electrical activity of the rat primary somatosensory cortex

2.1 Effects of isoflurane on the spontaneous electrical activity of the rat primary somatosensory cortex

In the next part of the research the effect of gas anesthetic isoflurane on the electrical activity in the rat S1 vibrissae representation during the first week of life was analyzed. The electrical activity in control conditions was characterized by bursts occurring at frequency $5.6 \pm 1.6 \text{ min}^{-1}$ including oscillations at alpha-beta and gamma frequency ranges associated with multiple unit activity (P2-7, $n = 6$) (Fig. 5). Isoflurane affected spontaneous gamma oscillations and spindle-bursts in a concentration-dependent manner (Fig. 5 A). While at 0.5% of isoflurane, the frequency of bursts and multiple unit activity were not significantly modified, at surgical anesthesia levels (1.5–2%) isoflurane completely suppressed the spontaneous spindle-bursts and gamma oscillations, which were associated with a reduction of oscillation power in alpha-beta (8–30 Hz) and gamma (30–80 Hz) ranges from $21 \pm 4 \mu\text{V}^2/\text{Hz}$ to $3 \pm 1 \mu\text{V}^2/\text{Hz}$ and $2.6 \pm 0.7 \mu\text{V}^2/\text{Hz}$ to $0.6 \pm 0.1 \mu\text{V}^2/\text{Hz}$, respectively ($n = 6$; $p < 0.01$) (Fig. 5). Suppression of activity was accompanied by almost complete disappearance of multiple unit activity with a drop in its frequency from $5.5 \pm 2.3 \text{ s}^{-1}$ to $0.1 \pm 0.1 \text{ s}^{-1}$ (Fig. 5).

To determine the age dependence of isoflurane (1.5-2%) effects, animals of the following age groups were used: P2-7, P8-15 and P16-69 (Fig. 5B). In P2-5 rats isoflurane exposure led to complete suppression of the spontaneous activity, however, by the end of the first week of life rare ($0.7 \pm 0.3 \text{ min}^{-1}$) spontaneous sharp activity (amplitude $226 \pm 123 \mu\text{V}$; half-duration $20 \pm 10 \text{ ms}$), accompanied by discharges of individual neurons ($n = 2$) were apparent.

Since the second postnatal week spontaneous activity in control has become continuous. In the presence of isoflurane there were isolated sharp potentials (amplitude $797 \pm 219 \mu\text{V}$) occurred at $6.6 \pm 2.6 \text{ min}^{-1}$ and synchronized most of multiple unit activity (P8-15, $n = 5$). These sharp potentials increased with age in frequency and amplitude. In contrast to newborn rats, in which isoflurane almost completely inhibited electrical activity, sharp potentials and bursts maintained the frequency of multiple unit activity at significant levels in older animals ($2.0 \pm 0.7 \text{ s}^{-1}$ at P8-15 and $8.4 \pm 3.2 \text{ s}^{-1}$ at P16- 69). Starting from the third postnatal week

isoflurane induced burst-suppression patterns (Ferron et al., 2009; Amzica et al., 2009) consisted of high-amplitude activity period and accompanied by a period of isoelectric EEG with an occurrence of $23.5 \pm 6.6 \text{ min}^{-1}$ and intraburst frequency of $12.5 \pm 3.0 \text{ Hz}$ (P16-69, $n = 9$).

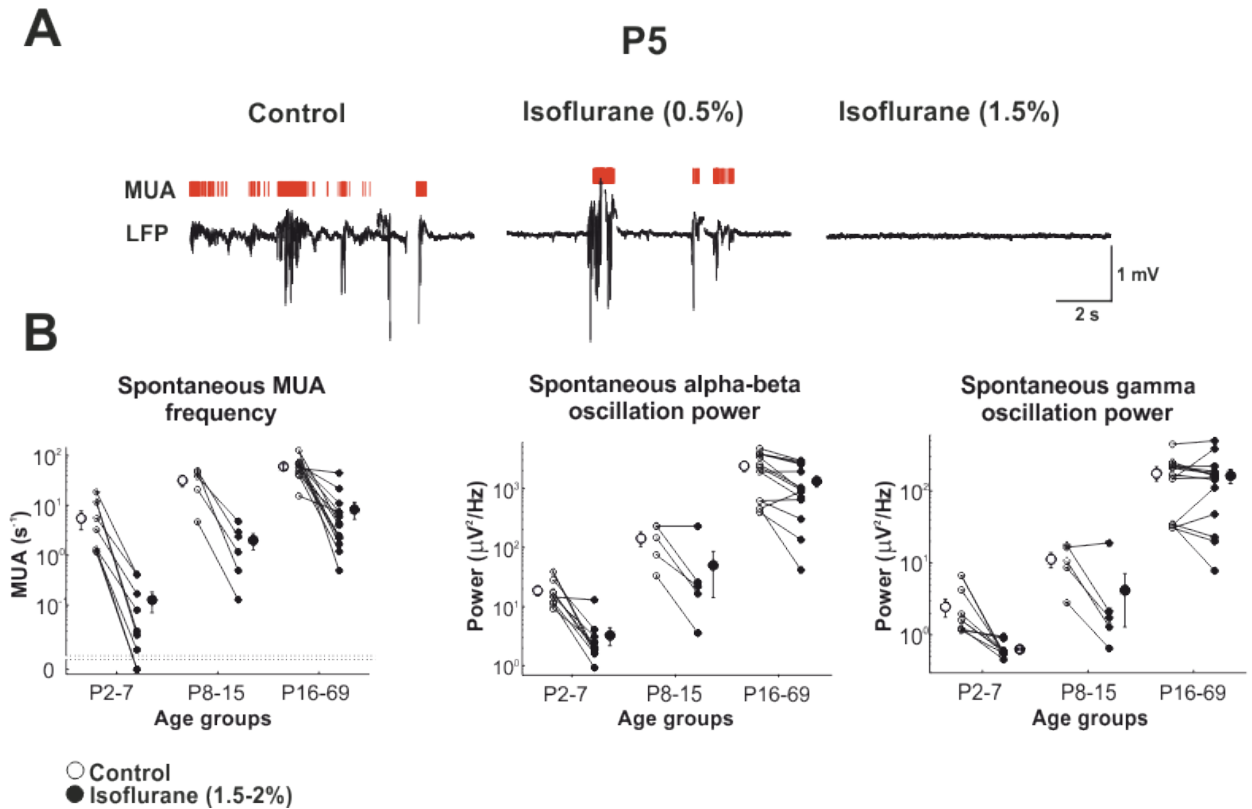


Figure 5 – Effects of isoflurane (0.5-2%) on the spontaneous activity in the whiskers representation in the S1 of the newborn rat. (A) Example traces of the spontaneous electrical activity in the L4 cortical column of the P5 rat brain (LFP – local field potentials – black lines; MUA – multiple unit activity – red vertical lines above) in control conditions (left) and after isoflurane inhalation in a concentration of 0.5% (center) and 1.5% (right). (B) Age dependence of isoflurane effects (1.5-2%) on the parameters of the spontaneous electrical activity in rat brain.

2.2 Effects of isoflurane on the sensory evoked electrical activity of the rat primary somatosensory cortex

During the first week of life isoflurane not only suppressed spontaneous activity but also completely blocked sensory evoked oscillations. The decrease in the power of sensory evoked oscillations (both gamma and spindle-burst) depended on concentration; complete suppression was observed when applying isoflurane at a concentration of 1.5-2% (Fig. 6).

In P2-7 rats analysis of experiments revealed that isoflurane causes a reduction in power of the evoked gamma and alpha-beta oscillations from 46 ± 17

$\mu\text{V}^2/\text{Hz}$ to $0.8 \pm 0.2 \mu\text{V}^2/\text{Hz}$ and from $237 \pm 96 \mu\text{V}^2/\text{Hz}$ to $8 \pm 3 \mu\text{V}^2/\text{Hz}$, respectively ($n = 6$, $p < 0.01$) (Fig. 6 A); this was accompanied by a reduction in multiple unit activity count from 35.6 ± 13.8 to 0.9 ± 0.5 units ($n = 6$, $p < 0.01$) (Fig. 6 A). The sensory evoked potentials amplitudes in control conditions and in the presence of isoflurane remained unchanged and were of $601 \pm 158 \mu\text{V}$ and $700 \pm 154 \mu\text{V}$, respectively ($n = 6$) (Fig. 6 B). Unit count during the sensory evoked potentials was also unchanged.

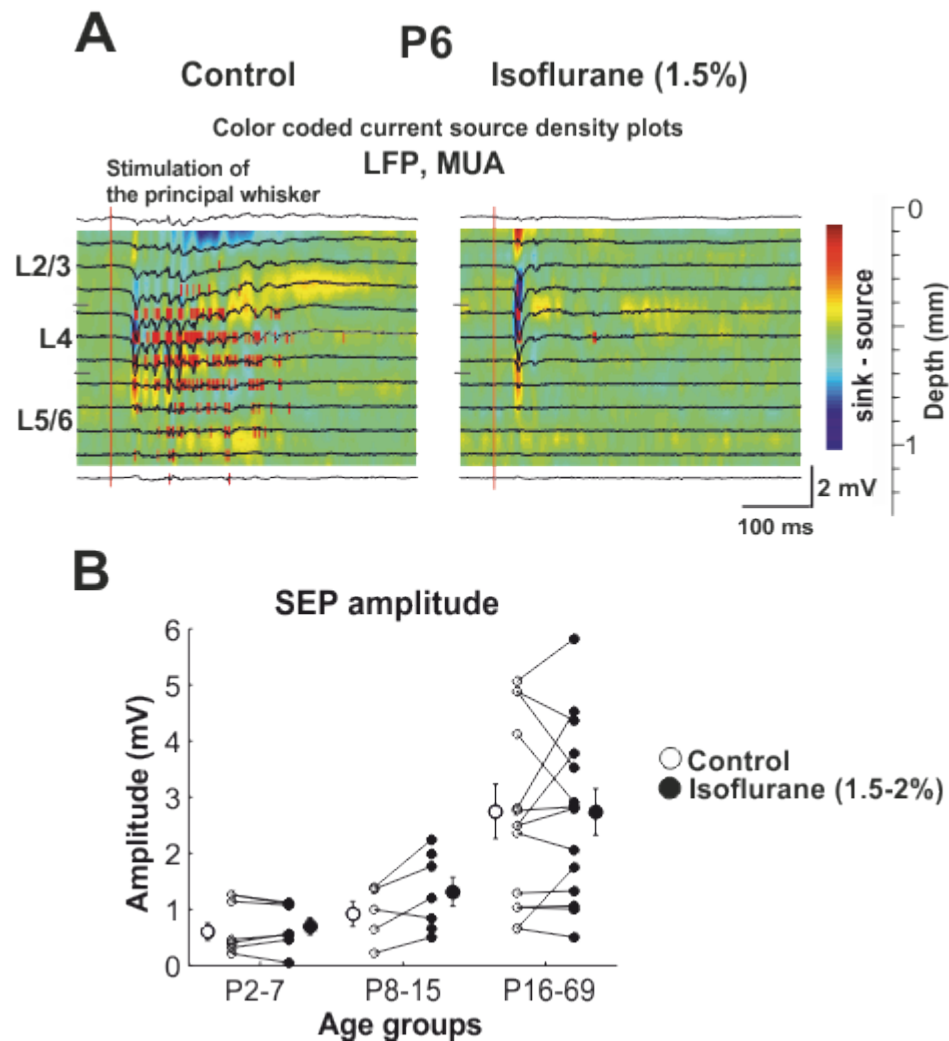


Figure 6 – Effects of isoflurane (1.5%) on the sensory evoked responses in the whiskers representation in the S1 of the newborn rat. Example traces of the sensory evoked responses caused by mechanical stimulation of the principal whisker at different cortical depths of the P6 rat brain (LFP – local field potentials – black traces; MUA – multiple unit activity – red vertical lines overlaid on the color coded current source density plot) under control conditions (left) and after inhalation of isoflurane (right). (B) Age dependence of isoflurane effects (1.5-2%) on amplitudes of SEP – sensory evoked potentials. Each pair corresponds to an individual rat; circles with error bars indicate the group averages at P2–7, P8–15, and P16–69.

Thus, the obtained data indicate that at surgical levels of anesthesia (1.5-2%) isoflurane causes complete suppression of the spontaneous activity, as well as the oscillatory component caused by sensory stimulation in the rat S1 during the first week after birth, while in older animals the anesthetic exposure induced burst-suppression patterns.

2.3 The study of combined administration of ketamine and midazolam effects on the electrical activity of the primary somatosensory cortex of newborn rats

To analyze the impact of combined treatment with ketamine and midazolam on electrical activity of the cortex of neonatal rats, these substances were administered (IP) at concentrations equivalent to the doses used for surgical anesthesia: 40 mg/kg and 9 mg/kg, respectively. The registration of electrical activity was carried out in the area of forelimbs representation.

Electrical activity in the somatosensory cortex of newborn rats under control conditions was characterized by bursts (with a frequency of $5.2 \pm 1.2 \text{ min}^{-1}$ and a duration of $2155 \pm 416 \text{ ms}$) including oscillations in gamma and alpha-beta frequency ranges associated with multiple unit activity (P5-7, $n = 6$) (Fig. 7 A). Combined administration (IP) of ketamine (40 mg/kg) and midazolam (9 mg/kg) significantly suppressed the spontaneous electrical activity during the first minutes after the injection (Fig. 7) until its disappearance in the majority of the experiments. The mean frequency of multiple unit activity decreased from 2.5 ± 0.7 to $0.2 \pm 0.1 \text{ s}^{-1}$ (to $11 \pm 5\%$) ($n = 6$, $p < 0.01$) and the frequency of bursts reduced from 5.2 ± 1.2 to $0.2 \pm 0.1 \text{ min}^{-1}$ (to $6 \pm 4\%$) ($n = 6$, $p < 0.01$). Analysis of the spontaneous motor activity mechanogram showed that the frequency of myoclonic movements decreased to $10 \pm 3\%$ (in control: 10.5 ± 1.3 , after injection of anesthetics: $1.0 \pm 0.4 \text{ min}^{-1}$) ($n = 6$, $p < 0.01$). Deep suppression of cortical activity lasted for 3 h after anesthetics administration (Fig. 7).

The effect of combined administration of the investigated anesthetics on the sensory evoked activity was less pronounced. Changes in the amplitude and latency of the sensory evoked potentials were unreliable. However, the oscillatory component of the sensory evoked response was significantly reduced to control: the oscillation power in gamma frequency decreased to $19 \pm 4\%$ ($n = 6$, $p < 0.01$), in the alpha-beta frequency range – to $14 \pm 4\%$ ($n = 6$, $p < 0.01$). The frequency of multiple unit activity occurring during the sensory evoked potentials decreased to $50 \pm 10\%$ ($n = 6$, $p < 0.01$), the frequency of multiple unit activity that occurred during the sensory evoked bursts reduced to $5 \pm 3\%$ ($n = 6$, $p < 0.01$) from control levels.

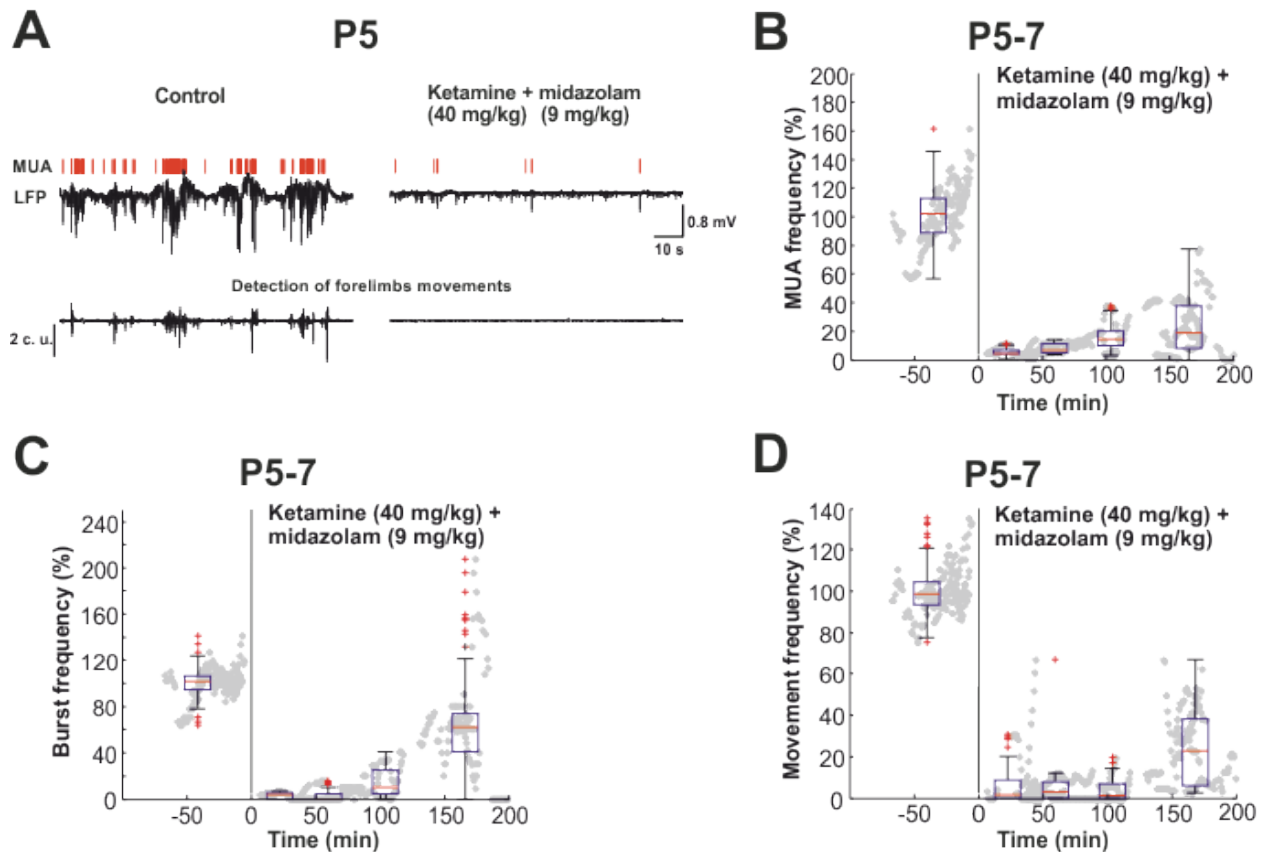


Figure 7 – Effects of ketamine (40 mg/kg) in combination with midazolam (9 mg/kg) on the spontaneous activity in the S1 of neonatal rats. (A) Example traces of electrical activity in the L4 of the forelimbs representation area in the S1 and mechanogram of the motor activity of the P5 rat (LFP – local field potentials – black traces; MUA – multiple unit activity – red vertical lines above; c. u. – conventional units) in control conditions (left) and 3 min after injection of ketamine in combination with midazolam (IP) (right). (B) The impact of combined administration of anesthetics on the frequency of multiple unit activity. (C) The impact of combined administration of anesthetics on the frequency of burst occurrence. (D) The effect of combined administration of anesthetics on the frequency of spontaneous movements. Each gray dot represents the moving average of the parameter. The vertical line indicates the time of anesthetics combination injection (IP). Points before time moment $t = 0$ correspond to control conditions. The pooled data obtained in the area of forelimbs representation of P5-7 rats normalized to control values are presented ($n = 6$).

Thus, the obtained data indicate that combination of ketamine (40 mg/kg) and midazolam (9 mg/kg) in clinically relevant doses used for surgical anesthesia causes almost complete suppression of the spontaneous electrical activity, as well as a significant decrease in the oscillatory component of the sensory evoked response in the S1 of newborn rats.

CONCLUSION

The comparative study of age and concentration dependences of ethanol impact on the activity of the primary somatosensory cortex and its neuroapoptogenic effects revealed that during the period of the most powerful neurotoxic effect (P4-7) ethanol completely suppresses the work of the primary somatosensory cortex. It was also shown that the dose dependence of ethanol neuroapoptotic action in this age group correlates with its inhibitory effect on the neuronal activity. The results of this study suggest a direct link between the neuroapoptotic effects of ethanol and the suppression of neuronal activity during the critical period of development.

Inhalation of isoflurane, as well as administration of combination of general anesthetics ketamine and midazolam in clinically relevant concentrations throughout the first postnatal week, lead to complete inhibition of spontaneous cortical activity and sensory evoked oscillations, while in more adult animals isoflurane causes burst-suppression patterns.

Effects of ethanol and general anesthetics, including isoflurane, ketamine, midazolam, are associated with modulation of a number of ion channels and receptors, including blocking of NMDA receptors and activation of GABA-A receptors that leads to inhibition of electrical activity of the developing brain. It is known that the viability of many central and peripheral neurons is critically dependent on electrical activity, which suppresses apoptotic mechanisms, increases antioxidant defense and also activates Ca^{2+} dependent regulatory processes for the expression of proapoptotic genes (Papadia et al., 2008; Papadia et al., 2005). Therefore, it can be assumed that the neuroapoptotic effects of ethanol and anesthetics are based on the suppression of cortical activity that is specific for early age and reflects the unique and high sensitivity of the mechanisms of early activity patterns generation to alcohol and general anesthesia.

In accordance with the obtained results it can be concluded that ethanol, as well as general anesthetics that are widely used in clinical practice including pediatrics inhibit the electrical activity of the fetal brain and can have adverse neurodegenerative effects on the intrauterine development process that can subsequently lead to the occurrence of various disturbances.

FINDINGS

1. During the first week after birth ethanol suppresses the spontaneous and the sensory evoked oscillatory bursts of activity, as well as induces apoptosis of neurons in the rat primary somatosensory cortex in a dose-dependent manner (in concentrations from 1 to 6 g/kg).
2. Concentration dependence of ethanol neuroapoptogenic effects during the critical period of development of the rat primary somatosensory cortex (from 4 to 7 postnatal days) correlates with the degree of inhibition of electrical activity.
3. Ethanol at a concentration of 6 g/kg during the third week after birth slows down the ongoing background EEG activity of the rat primary somatosensory cortex causing slow delta wave oscillations and decreasing the activity of neurons in all layers.
4. Isoflurane in a concentration of 1.5-2% corresponding to the level of surgical anesthesia completely suppresses the spontaneous early gamma and spindle-burst oscillations and also blocks the sensory evoked oscillatory responses in the rat primary somatosensory cortex during the first week of postnatal development.
5. As a result of isoflurane (1.5-2%) exposure during the third week of life burst-suppression patterns alternating with activity suppression periods arise in the rat primary somatosensory cortex.
6. Combined administration of clinically relevant doses of general anesthetics – ketamine (40 mg / kg) and midazolam (9 mg / kg) used for surgical anesthesia leads to a nearly complete suppression of the spontaneous electrical activity, as well as a decrease in the oscillatory component of the sensory evoked response in the primary somatosensory cortex of newborn rat brain during the first postnatal week.

LIST OF PUBLISHED WORKS ON THE THEME OF DISSERTATION

Articles in scientific journals included in Web of Science citation system and recommended by Higher Attestation Commission

- 1) Inhibition of Cortical Activity and Apoptosis Caused by Ethanol in Neonatal Rats In Vivo / **Lebedeva J.**, Zakharov A., Ogievetsky E., Minlebaeva A., Kurbanov R., Gerasimova E., Sitdikova G., Khazipov R. // Cerebral Cortex. – New York, USA. – 2015. – P 1-15. – DOI: 10.1093/cercor/bhv293. – 0,88 printed sheets (personally by the author – 0,45).
- 2) Ketamine-Midazolam Anesthesia Induces Total Inhibition of Cortical Activity in the Brain of Newborn Rats / **Lebedeva Yu.A.**, Zakharova A.V., Sitdikova G.F., Zefirov A.L., Khazipov R.N. // Bulletin of Experimental Biology and Medicine. – Moscow, Russia. – 2016. – V. 161, Is. 1. – P 15-19. – DOI: 10.1007/s10517-016-3334-1. – 0,25 printed sheets (personally by the author – 0,16).
- 3) Gamma Oscillations in the Somatosensory Cortex of Newborn Rats / Gerasimova E.V., Zakharov A.V., **Lebedeva Yu.A.**, Inacio A.R. , Minlebaev M.G., Sitdikova G.F., Khazipov R.N. // Bulletin of Experimental Biology and Medicine. – Moscow, Russia. – 2014. – V. 156, Is. 3. – P. 295-298. – DOI: 10.1007/s10517-014-2333-3. – 0,19 printed sheets (personally by the author – 0,04).
- 4) Isoflurane suppresses early cortical activity / Sitdikova G., Zakharov A., Janackova S., Gerasimova E., **Lebedeva J.**, Inacio A. R., Zaynutdinova D., Minlebaev M., Holmes G. L., Khazipov R. // Annals of Clinical and Translational Neurology. – USA. – 2014. – V. 1, Is. 1. – P. 15-26. – DOI: 10.1002/acn3.16. – 0,69 printed sheets (personally by the author – 0,15).
- 5) Sensory feedback synchronizes motor and sensory neuronal networks in the neonatal rat spinal cord / Ina'cio A.R., Nasretudinov A., **Lebedeva J.**, Khazipov R. // Nature Communications. – England. – 2016. – V. 7: 13060. – P. 1-14. – DOI: 10.1038/ncomms13060. – 0,88 printed sheets (personally by the author – 0,16).

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